ENHANCED SECRETION OF CYCLODEXTRIN GLUCANOTRANSFERASE IN *Lactococcus lactis* USING HETEROLOGOUS SIGNAL PEPTIDE AND OPTIMIZATION OF INDUCTION CONDITION FOR CULTIVATION

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A thesis submitted in fulfilment of the
requirements for the award of the degree of
Master of Engineering (Bioprocess)

Faculty of Chemical Engineering
Universiti Teknologi Malaysia

MAY 2013

"Terima kasih Mak, kakak, sahabat dan semua yang terlibat. Ingatan tulus ikhlas untuk Allahyarham ayah yang tersayang, Mahmud bin A. Rahman"

ACKNOWLEDGEMENT

In the name of Allah, The Most Compassionate and The Most Benevolence who bestowed me the enlightment, the truth, the knowledge and with regards to Prophet Muhammad S.A.W for the guidance to the straight path. I thank to Allah for giving me the strength in completing this thesis. May Allah bless me with the ability to continue the good deeds to the community in this field. Many people have parts in this text I did as a writer.

Firstly, I would like to express my sincere gratitude and great appreciation to my supervisor, Prof Dr Rosli Md Illias and my co-supervisor, Prof Raha Abdul Rahim for their continuous support, encouragement and contribution, either directly or indirectly in making this thesis.

Special appreciation to my beloved mother and all siblings for their continous encouragement and pray. My acknowledgement also goes to all my labmate for their encouragements and smiles Without them, this thesis would not have been the same as presented here. I would also extend my gratitude to Nor Hasmaliana binti Abdul Manas for her contribution and help reviewing this text .

ABSTRACT

Protein secretion is preferable compared to intracellular production due to its easy subsequent purification process. The secretion generally requires a particular N-terminal signal peptide to lead the precursor protein to the secretion machinery. In this study, a strategy to secrete a cyclodextrin glucanotransferase (CGTase) from *Bacillus* sp G1 into the culture medium of *Lactococcus lactis* using three different signal peptides was developed. Heterologous signal peptides which are G1 (native signal peptide of CGTase from *Bacillus* sp G1) and M5 (mutated form of G1 signal peptide by introduction of helix breaker at H-region signal peptides) were used for inducible and secretory expression of CGTase in *L. lactis*. The effectiveness of these heterologous signal peptides was compared to the homologous signal peptides which is SPUsp45 signal peptide (derived from Unknown Secreted 45 kDa Protein of *L. lactis*). Secretion activity of CGTase led by G1 signal peptide was significantly increased by 46.2% and 75.0% compared to CGTase fused to M5 and SPUsp45 signal peptide, respectively after 6 hour post-induction. Sequence analysis showed there is no correlation between signal peptide characteristics (N-terminal signal peptide, hydrophobic signal peptide and C-terminal cleavage site) and secretion level of CGTase. In addition, Response Surface Methodology (RSM) was applied to CGTase led by G1 signal peptide (G1-CGTase) to optimize culture cultivation for post induction temperature, nisin concentration and inducer starting point (OD_{600}). The G1-CGTase activity increased approximately 2.81 fold from 5.79 U/mL to 16.89 U/mL at the optimized post induction temperature, nisin concentration and inducer starting point (OD_{600}) of 20.1°C, 3.086 ng/mL and 0.09, respectively. Hence, G1 signal peptide has a great potential to be incorporated in an expression vector to increase the level of recombinant protein secretion in *L. lactis.*

ABSTRAK

Rembesan protein menjadi pilihan berbanding penghasilan intraselular kerana dapat memudahkan proses penulenan. Umumnya, rembesan protein memerlukan turutan N-terminal yang dinamakan peptida isyarat untuk membawa protein pelopor kepada jentera rembesan. Dalam kajian ini, satu strategi untuk merembeskan siklodekstrin glucanotransferase (CGTase) daripada *Bacillus* sp G1 ke dalam medium kultur *Lactococcus lactis* menggunakan tiga peptida isyarat yang berbeza telah dibangunkan. Peptida isyarat heterologous iaitu G1 (peptide isyarat asal CGTase daripada *Bacillus* sp G1) dan M5 (mutan peptida isyarat G1 dengan pengenalan helix terpecah pada kawasan H peptida isyarat) telah digunakan untuk induksi dan merembeskan CGTase dalam *L. lactis*. Kecekapan peptida isyarat heterologous telah dibandingkan dengan peptida isyarat homolog iaitu SPUsp45 (diperolehi daripada "Unknown Secreted 45 kDa Protein" dalam *L. lactis*). Rembesan CGTase yang dibawa oleh peptida isyarat G1 telah meningkat dengan ketara sebanyak masing-masing pada 46.2% dan 75% dibandingkan dengan CGTase yang digabungkan dengan peptida isyarat M5 dan peptida isyarat SPUsp45 selepas 6 jam induksi. Jujukan analysis menunjukkan tiada hubungkait di antara ciri-ciri peptida isyarat (N-terminal, hidrofobik dan C-terminal tapak belahan) dengan tahap rembesan CGTase. Selain itu, kaedah gerak balas permukaan (RSM) telah digunakan oleh CGTase yang dibawa oleh peptida isyarat G1 untuk mengoptimumkan suhu selepas induksi, kepekatan nisin dan titik aruhan permulaan (OD_{600}). Aktiviti G1-CGTase meningkatkan sebanyak 2.8 kali ganda daripada 5.79 U/mL kepada 16.89 U/mL untuk suhu optima selepas induksi, kepekatan nisin dan titik aruhan permulaan (OD_{600}) masing-masing pada 20.1°C, 3.086 ng/mL dan 0.09. Oleh itu, peptida isyarat G1 mempunyai potensi yang besar untuk dimasukkan ke dalam vektor ungkapan bagi meningkatkan tahap rembesan protein rekombinan dalam *L. lactis*.

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LIST OF SYMBOLS

%	-	Percent
µm	-	micromolar
bp	-	basepair
cm	-	centimeter
g/L	-	gram per liter
kDa	-	kiloDalton
kV	-	kiloVolt
M	-	Molar mass
mg	-	milligram
mg/mL	-	milligram per mililiter
min	-	minutes
mL	-	milliliter
mM	-	millimolar
nm	-	nanometer
ºC	-	Degree Celcius
OD_{600}	-	Optical density at 600nm
rpm	-	revolutions per minutes
U	-	Unit
V	-	Volt
v/v	-	Volume per volume
w/v	-	Weight per Volume
β-CD	-	Beta Cyclodextrin
µg/mL	-	microgram per milliliter
µL	-	microliter

LIST OF ABBREVIATION

a.a	-	amino acid
ABC transporter	-	ATP- binding cassette transporter
AmyQ	-	Amylase
ATP	-	Adenosine-5-triphosphate
B. subtilis	-	*Bacillus subtilis*
BSA	-	Bovine serum albumin
$CaCI_2$	-	Calcium Chloride
CAT	-	Chloramphenicol acetyltransferase
CCD	-	Central composite design
CD	-	Cyclodextrin
CGTase	-	Cyclodextrin glucanotransferase
Chl	-	Chloramphenicol
CWA	-	Cell wall anchored
ddH_2O	-	deionized distilled water
DNA	-	Deoxyribonucleic acid
dNTP	-	Deoxyribonucleotide triphosphate
dNTPs	-	Deoxynucleotide triphosphate
DTT	-	Dithiothreitol
E. coli	-	*Escherichia coli*
EDTA	-	Ethylenedianetetra-acetate
EtBr	-	Ethidium bromide
Ffh	-	Fifty four homolog
GRAS	-	Genarally regarded as safe
HCl	-	Hydrogen chloride
L. lactis	-	*Lactococcus lactis*

LB	-	Luria Bertani
LysM	-	Lysine Motif
$MgCI_2$	-	Magnesium chloride
$MgSO_4$	-	Magnesium sulfide
Na_2CO_3	-	Natrium carbonate
NaOH	-	Natrium hydroxide
NICE	-	NIsin Controlled Expression
Nuc	-	Nuclease
OFAT	-	One-factor-at-a-time
PAGE	-	Polyacrylamide Gel Electrophoresis
PCR	-	Polymerase chain reaction
PMSF	-	Phenylmethlysulfonylfluride
PPIase	-	peptidyl-propyl-*cis/trans*-isomerase
RNA	-	Ribonucleic acid
RNase	-	Ribonuclease
RSM	-	Response surface methodology
S. aureus	-	*Staphylococcus aureus*
ScRNA	-	Small cytoplasmic RNA
SDS	-	Sodium Dodecyl Sulphate
Sec	-	Secretory
SP	-	Signal peptide
SPase	-	Signal peptidase
SPPase	-	Signal peptide peptidase
SRP	-	Signal recognition particles
TAE	-	Tris-acetic acid-EDTA
TCA	-	Tricholoroacetic acid
TE	-	Tris-EDTA

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CHAPTER 1

INTRODUCTION

1.0 Introduction

Efficient protein secretion is very important in biotechnology as it provides an active and stable enzyme production, which is essential for successful biocatalysis. Secretion is always preferable to cytoplasmic production due to its several advantages such as providing N-terminal authenticity of the expressed protein, allowing continuous culture (Lv *et al.*, 2012), simplifing purification process, avoiding proteolysis, enhancing biological activity and giving high product stability and solubility (Mergulhão *et al.*, 2005).

For many decades, numerous attempts were made to improve the secretion efficiency of extracellular protein production in bacteria. *Escherichia coli*, at present, is the dominant prokaryotic system used for industrial gene expression due to its well established genetic tools, ease in genetic handling, long-term experience and extensive documentation with the US Food and Drug Administration and other regulatory bodies. However, the high production of enzyme in the cytoplasm subsequently leads to the formation of inclusion bodies. Thus, the

secretion of enzyme to the extracellular milieu is a new approach to overcome such problem. However, the extracellular secretion in *E. coli* though always correlates with no-specific leakage and cell lyses (Ismail *et al.*, 2011).

In contrast to Gram-negative bacteria such as *E. coli*, an increase of interest has been shown to *Lactococcus lactis* as an expression host for recombinant protein production. It has a number of advantages over conventional cell factories like *E. coli* and *B. subtilis*. The bacterium has a well established safety profile and a Generally Regarded as Safe (GRAS) status. This feature makes it suitable to be used as delivery vehicles in pharmaceuticals and in industrial manufactures of fermented food product (Liang *et al*., 2007). It is Gram positive bacterium and therefore does not posses endotoxic lipopolysaccharides (LPS) which are associated with Gram negative bacteria. Moreover, experimental data and genomic analyses indicate that only one major protein, Usp45, is secreted into the medium thus simplifying downstream purification processes (van Asseldonk *et al*., 1993). In addition, *L. lactis* laboratory strains possess only 1 exported housekeeping protease, HtrA .

Recently, many studies concerning the potential of *L. lactis* as a cell factory for production and secretion of recombinant proteins have been carried out. However, low secretion level of heterologous proteins by *L. lactis* becomes a bottleneck for its application in industry. Therefore, numerous genetic tools and modifications have been developed to enhance the secretion efficiency in *L. lactis* such as 1) overexpression of intracellular chaperone for secretion competency (Martinez-Alonso *et al.*, 2010), 2) fusion of protein of interest to a heterologous or homologous signal peptide for translocation recognition (Ravn et al., 2003, Ng and Sarkar, 2012), 3) implantation of secretion machinery to improve secretion translocation (Nouaille *et al.*, 2006) and 4) over-expression of extracellular chaperone to improve folding of the secreted protein (Lindholm *et al.*, 2006). In addition, cultivation strategies are also identified as a factor that contributes to the extracellular secretion of the recombinant proteins in *L. lactis* host cell. The culture medium composition, temperature, pH, or medium supplements are important

parameters that might influence the extracellular expression of the recombinant protein in *L. lactis* (Berlec *et al*., 2008).

Among all, the second strategy is commonly used to enhance the secretion efficiency in *L. lactis*. Most secreted proteins are synthesized as precursors with N-terminal signal peptide and the mature moiety of the protein. Precursors are recognized by secretion machinery and translocated across the membrane. The signal peptide is removed by a signal peptidase after translocation occurs and subsequently releases the mature protein into the medium. Thus, the selection of an optimal signal peptide is important for efficient secretory production of recombinant proteins. The most commonly used signal peptides for the heterologous protein production in *L. lactis* is Usp45 signal peptide (Asseldonk *et al*., 1993). The Usp45 signal peptide is a homologous signal peptide isolated from the genome of *L. lactis* MG1363. Previous studies showed that both natural signal peptides (e:g SP310 and SP_{EXP4}) and engineered signal peptides (e;g SP310mut2) have secretion efficiency only as good as and often worse than Usp45 (Ravn *et al.*, 2000, Ravn *et al.*, 2003, Morello *et al.*, 2008). Therefore, it is a challenge to find an optimal signal peptide that can improve the secretion efficiency of recombinant protein in *L. lactis*

A significant finding by Jonet *et al.* (2012) on extracellular secretion in *E. coli* showed that utilization of a heterologous signal peptide of CGTase (G1 signal peptide) from *Bacillus* sp G1 can improve the secretion of heterologous protein into extracellular space. Furthermore, the engineered signal peptide called M5 has proven to confer a higher secretion level of recombinant CGTase than G1 signal peptide. Hence, these signal peptides might have the potential to be employed as an alternative signal peptide in *L. lactis*.

In this study, the effect of signal peptides in protein secretion by *L. lactis* was investigated. The heterologous signal peptide (G1), engineered signal peptide (M5) and homologous signal peptide (Usp45) were used. In addition, CGTase mature gene was chosen as the model protein for secretion using these signal peptides.

Furthermore, this study also describes the optimization of cultivation conditions using statistical modeling in order to enhance protein production and secretion in *L. lactis*.

1.2 Objective

The objectives of this project are to study the effectiveness of using heterologous signal peptides and the optimization of cultivation conditions on CGTase production in *L. lactis*

1.3 Scope of the study

The scopes of the study are:

a) Cloning of G1, M5 and Usp45 signal peptides fused with CGTase mature gene.
b) Comparison of secretion efficiency directed by G1, M5 and Usp45 signal peptides and signal peptide sequence analysis.
c) To study the effects of different cultivation conditions which are post induction temperature, nisin concentration and inducer starting point on protein secretion.
d) Optimization of cultivation conditions of CGTase in *L. lactis* using response surface methodology (RSM).

REFERENCES

Adams, H., Scotti, P. A., de Cock, H., Luirink, J. and Tommassen, J. (2002). The presence of a helix breaker in the hydrophobic core of signal sequences of secretory proteins prevents recognition by the signal-recognition particle in *Escherichia coli*. *European Journal of Biochemistry.* 269 (22),5564-5571.

Aiba, S., Humphrey, A.E., and Millias, N.F.(1973). *Biochemical Engineering*. 2nd Edition. Academic Press. New York.92-127

Akita, M., Sasaki, S., Matsuyama, S. andMizushima, S. (1990). SecA interacts with secretory proteins by recognizing the positive charge at the amino terminus of the signal peptide in Escherichia coli. *Journal of Biological Chemistry,* 265**,** 8164-9.

Asseldonk, M., Vos, W. M. and Simons, G. (1993). Functional analysis of the *Lactococcus lactis* usp45 secretion signal in the secretion of a homologous proteinase and a heterologous α-amylase. *Molecular and General Genetics MGG.* 240 (3),428-434.

Azaman, S. N. A., Ramakrishnan, N. R., Tan, J. S., Rahim, R. A., Abdullah, M. P. and Ariff, A. B. (2010). Optimization of an induction strategy for improving interferon-α2b production in the periplasm of *Escherichia coli* using response surface methodology. *Biotechnology and Applied Biochemistry.* 56 (4),141-150.

Bahey-El-Din, M., Casey, P. G., Griffi n, B. T. and Gahan, C. G. (2010). Expression of two Listeria monocytogenesantigens (P60 and LLO) in *Lactococcus lactis* and examination for use as live vaccine vectors. *Journal Medical Microbiology*. 59(8), 904-912

Berlec, A., Tompa, G., Slapar, N., Fonović, U. P., Rogelj, I. and Štrukelj, B. (2008). Optimization of fermentation conditions for the expression of sweet-tasting

protein brazzein in *Lactococcus lactis*. *Letters in Applied Microbiology.* 46 (2),227-231.

Bermúdez-Humarán, L. G., Langella, P., Cortes-Perez, N. G., Gruss, A., Tamez-Guerra, R. S., Oliveira, S. C., Cardenas, O. S.-., Montes de Oca-Luna, R. and Le Loir, Y. (2003). Intranasal Immunization with Recombinant *Lactococcus lactis* Secreting Murine Interleukin-12 Enhances Antigen-Specific Th1 Cytokine Production. *Infection and Immunity.* 71 (4),1887-1896.

Bermúdez-Humarán, L. G., Langella, P., Miyoshi, A., Gruss, A., Guerra, R. T., Montes de Oca-Luna, R. and Le Loir, Y. (2002). Production of Human Papillomavirus Type 16 E7 Protein in *Lactococcus lactis*. *Applied and Enviroment Microbiology* 68 (2),917-922.

Bolhuis, A., Broekhuizen, C. P., Sorokin, A., van Roosmalen, M. L., Venema, G., Bron, S., Quax, W. J. and van Dijl , J. M. (1998). SecDF of *Bacillus subtilis*, a Molecular Siamese Twin Required for the Efficient Secretion of Proteins. *Journal of Biological Chemistry.* 273 (33),21217-21224.

Borrero, J., Jiménez, J. J., Gútiez, L., Herranz, C., Cintas, L. M. and Hernández, P. E. (2011). Protein expression vector and secretion signal peptide optimization to drive the production, secretion, and functional expression of the bacteriocin enterocin A in lactic acid bacteria. *Journal of Biotechnology.* 156 (1),76-86.

Biro, J. C. (2006). Amino acid size, charge, hydropathy indices and matrices for protein structure analysis. *Theoretical Biology and Medical Modelling,* 3**,** 1-12.

Brockmeier, U., Caspers, M., Freudl, R., Jockwer, A., Noll, T. and Eggert, T. (2006). Systematic Screening of All Signal Peptides from *Bacillus subtilis*: A Powerful Strategy in Optimizing Heterologous Protein Secretion in Gram-positive Bacteria. *Journal of Molecular Biology.* 362 (3),393-402.

Chen, M. and Nagarajan, V. (1994). Effect of alteration of charged residues at the N termini of signal peptides on protein export in *Bacillus subtilis*. *Journal of Bacteriology.* 176 (18),5796-5801.

Choo, K. and Ranganathan, S. (2008). Flanking signal and mature peptide residues influence signal peptide cleavage. *BMC Bioinformatics,* 9**,** S15.

Corthier, G., Delorme, C., Ehrlich, S. D. and Renault, P. (1998). Use of Luciferase Genes as Biosensors To Study Bacterial Physiology in the Digestive Tract. *Applied and Enviroment Microbiology.* 64 (7),2721-2722.

de Ruyter, P. G., Kuipers, O. P. and de Vos, W. M. (1996). Controlled gene expression systems for *Lactococcus lactis* with the food-grade inducer nisin. *Applied and Environmental Microbiology.* 62 (10),3662-7.

Demain, A. L. and Vaishnav, P. (2009). Production of recombinant proteins by microbes and higher organisms. *Biotechnology Advances.* 27 (3),297-306.

Dieye, Y., Usai, S., Clier, F., Gruss, A. and Piard, J.C. (2001). Design of a Protein-Targeting System for Lactic Acid Bacteria. *Journal of Bacteriology.* 183 (14),4157-4166.

Douillard, F., O'Connell-Motherway, M., Cambillau, C. and van Sinderen, D. (2011). Expanding the molecular toolbox for *Lactococcus lactis*: construction of an inducible thioredoxin gene fusion expression system. *Microbial Cell Factories.* 10 (1),66.

Driessen, A. J. M., Manting, E. H. and van der Does, C. (2001). The structural basis of protein targeting and translocation in bacteria. *Natural Structural Molecular Biology.* 8 (6),492-498.

Drouault, S., Anba, J., Bonneau, S., Bolotin, A., Ehrlich, S. D. and Renault, P. (2002). The Peptidyl-Prolyl Isomerase Motif Is Lacking in PmpA, the PrsA-Like Protein Involved in the Secretion Machinery of *Lactococcus lactis*. *Applied and Environmental Microbiology.* 68 (8),3932-3942.

Drouault, S., Corthier, G., Ehrlich, S. D. and and Renault, P. (2000). Expression of the *Staphylococcus hyicus* Lipase in *Lactococcus lactis*. *Applied and Environmental Microbiology.* 66 (2),588–598.

Donovan, R. S., Robinson, C. W. and Glick, B. R. (1996). Review: Optimizing inducer and culture conditions for expression of foreign proteins under the control of the lac promoter. *Journal of Industrial Microbiology*. 16: 145-154

Eichenbaum, Z., Federle, M. J., Marra, D., de Vos, W. M., Kuipers, O. P., Kleerebezem, M. and Scott, J. R. (1998). Use of the Lactococcal nisA Promoter To Regulate Gene Expression in Gram-Positive Bacteria: Comparison of Induction Level and Promoter Strength. *Applied and Environmental Microbiology.* 64 (8),2763-2769.

Enouf, V., Langella, P., Commissaire, J., Cohen, J. and Corthier, G. (2001). Bovine Rotavirus Nonstructural Protein 4 Produced by *Lactococcus lactis* Is Antigenic and Immunogenic. *Applied and Environmental Microbiology.* 67 (4),1423-1428.

Fernandez, A., Horn, N., Wegmann, U., Nicoletti, C., Gasson, M. J. and Narbad, A. (2009). Enhanced Secretion of Biologically Active Murine Interleukin-12 by *Lactococcus lactis*. *Applied and Environmental Microbiology.* 75 (9),2996.

Foucaud-Scheunemann, C. and Poquet, I. (2003). HtrA is a key factor in the response to specific stress conditions in *Lactococcus lactis*. *FEMS Microbiology Letters.* 224 (1),53-59.

Geoffroy, M.-C., Guyard, C., Quatannens, B., Pavan, S., Lange, M. and Mercenier, A. (2000). Use of Green Fluorescent Protein To Tag Lactic Acid Bacterium Strains under Development as Live Vaccine Vectors. *Applied and Environmental Microbiology.* 66 (1),383-391.

Gennity, J., Goldstein, J. andInouye, M. (1990). Signal peptide mutants of *Escherichia coli*. *Journal of Bioenergetics and Biomembranes,* 22**,** 233-269.

Giuliano, M., Schiraldi, C., Marotta, M. R., Hugenholtz, J. and Rosa, M. (2004). Expression of Sulfolobus solfataricus α-glucosidase in *Lactococcus lactis*. *Applied Microbiology and Biotechnology.* 64 (6),829-832.

Hazebrouck, S., Pothelune, L., Azevedo, V., Corthier, G., Wal, J.-M. and Langella, P. (2007). Efficient production and secretion of bovine beta-lactoglobulin by *Lactobacillus casei*. *Microbial Cell Factories.* 6 (1),12.

Heijne, G. y. (1990). The Signal Peptide. *The Journal of Membrane Biology.* 115 195-201.

Herranz, C. and Driessen, A. J. (2005). Sec-mediated secretion of bacteriocin enterocinP by *Lactococcus lactis*. Applied Environmental Microbiology. 71 (4), 1959-1963.

Huibregtse, I. L., Marietta, E. V., Rashtak, S., Koning, F., Rottiers, P., David, C. S., van Deventer, S. J. and Murray, J. A. 2009. Induction of antigen-specific tolerance by oral administration of *Lactococcus lactis* delivered immunodominant DQ8-restricted gliadin peptide in sensitized nonobese diabetic Abo Dq8 transgenic mice. *Journal Immunology*. (183) 4: 2390-2396

Holo, H. and Nes, I. F. (1989). High-Frequency Transformation, by Electroporation, of *Lactococcus lactis* subsp. cremoris Grown with Glycine in Osmotically Stabilized Media. *Applied and Environmental Microbiology.* 55 (12),3119-3123.

Hong, F., Meinander, N. Q. and Jönsson, L. J. (2002). Fermentation strategies for improved heterologous expression of laccase in *Pichia pastoris*. *Biotechnology and Bioengineering.* 79 (4),438-449.

Ismail, N., Hamdan, S., Mahadi, N., Murad, A., Rabu, A., Bakar, F., Klappa, P. and Illias, R. (2011). A mutant L-asparaginase II signal peptide improves the secretion of recombinant cyclodextrin glucanotransferase and the viability of *Escherichia coli. Biotechnology Letters.* 33 (5),999-1005.

Jonet, M. A., Mahadi, N. M., Murad, A. M. A., Rabu, A., Bakar, F. D. A., Rahim, R. A., Low, K. O. and Illias, R. M. (2012). Optimization of a Heterologous Signal Peptide by Site-Directed Mutagenesis for Improved Secretion of Recombinant Proteins in *Escherichia coli*. *Journal of Molecular Microbiology and Biotechnology.* 22 (1),48-58.

Kim, M., Lee, J., Kim, H., Sohn, C. and Oh, T. (1999). Overexpression of cyclodextrin glycosyltransferase gene from *Brevibacillus brevis* in *Escherichia coli* by control of temperature and mannitol concentration. *Biotechnology Techniques.* 13 (11),765-770.

Kleerebezem, M., Beerthuyzen, M. M., Vaughan, E. E., Vos, W. M. and Kuipers, O. P. (1997). Controlled gene expression systems for Lactic acid bacteria: Transferable Nisin- Inducible Expression Cassettes for *Lactococcus, Leuconostoc,* and *Lactobacillus* spp. *Applied and Environmental Microbiology.* 63 (11),4581-4584.

Knoll, A., Bartsch, S., Husemann, B., Engel, P., Schroer, K., Ribeiro, B., Stöckmann, C., Seletzky, J. and Büchs, J. (2007). High cell density cultivation of recombinant yeasts and bacteria under non-pressurized and pressurized conditions in stirred tank bioreactors. *Journal of Biotechnology.* 132 (2),167-179.

Kylä-Nikkilä, K., Alakuijala, U. andSaris, P. E. J. (2010). Immobilization of *Lactococcus lactis* to cellulosic material by cellulose-binding domain of Cellvibrio japonicus. *Journal of Applied Microbiology.*109**,** 1274-1283.

Larsen, N., Boye, M., Siegumfeldt, H. and Jakobsen, M. (2006). Differential Expression of Proteins and Genes in the Lag Phase of *Lactococcus lactis* subsp. lactis Grown in Synthetic Medium and Reconstituted Skim Milk. *Applied and Environmental Microbiology.* 72 (2),1173-1179.

Le Loir, Y., Azevedo, V., Oliveira, S., Freitas, D., Miyoshi, A., Bermudez-Humaran, L., Nouaille, S., Ribeiro, L., Leclercq, S. and Gabriel, J. (2005). Protein secretion in *Lactococcus lactis* : an efficient way to increase the overall heterologous protein production. *Microbial Cell Factories.* 4 2.

Le Loir, Y., Nouaille, S., Commissaire, J., Brétigny, L., Gruss, A. and Langella, P. (2001). Signal Peptide and Propeptide Optimization for Heterologous Protein Secretion in *Lactococcus lactis*. *Applied and Environmental Microbiology.* 67 (9),4119-4127.

Le loir. Y, A. Gruss, S. D. Ehrlich and Langella.,P. (1998). A Nine-Residue Synthetic Propeptide Enhances Secretion Efficiency of Heterologous Proteins in *Lactococcus lactis*. *Journal of Bacteriology.* 180 (7),1895-1903.

Lee, H. C. and Bernstein, H. D. (2001). The targeting pathway of *Escherichia coli* presecretory and integral membrane proteins is specified by the hydrophobicity of the targeting signal. *Proceedings of the National Academy of Sciences.* 98 (6),3471-3476.

Liang, L.X., Zhang, L., Zhong, J. and Huan, L. (2007). Secretory expression of a heterologous nattokinase in *Lactococcus lactis*. *Applied Microbiology and Biotechnology.* 75 (1),95-101.

Lindholm, A., Ellmén, U., Tolonen-Martikainen, M. and Palva, A. (2006). Heterologous protein secretion in *Lactococcus lactis* is enhanced by the;*Bacillus subtilis* chaperone-like protein PrsA. *Applied Microbiology and Biotechnology.* 73 (4),904-914.

Ling ,L, F., Zi Rong, X., Wei Fen, L., Jiang Bing, S., Ping, L. and Chun Xia, H. (2007). Protein secretion pathways in *Bacillus subtilis*: Implication for optimization of heterologous protein secretion. *Biotechnology Advances.* 25 (1),1-12.

Liu, H., Li, J., Du, G., Zhou, J. and Chen, J. (2012). Enhanced production of α-cyclodextrin glycosyltransferase in *Escherichia coli* by systematic codon usage optimization. *Journal of Industrial Microbiology & Biotechnology.* 1-9.

Llull, D. and Poquet, I. (2004). New Expression System Tightly Controlled by Zinc Availability in *Lactococcus lactis*. *Applied Microbiology and Biotechnology.* 70 (9),5398-5406.

Low, K., Muhammad Mahadi, N., Abdul Rahim, R., Rabu, A., Abu Bakar, F., Murad, A. and Md. Illias, R. (2011). An effective extracellular protein

secretion by an ABC transporter system in *Escherichia coli:* statistical modeling and optimization of cyclodextrin glucanotransferase secretory production. *Journal of Industrial Microbiology & Biotechnology.* 38 (9),1587-1597.

Low, K. O., Mahadi, N. M., Abdul Rahim, R., Rabu, A., Abu Bakar, F. D., Abdul Murad, A. M. and Md. Illias, R. (2010). Enhanced secretory production of hemolysin-mediated cyclodextrin glucanotransferase in *Escherichia coli* by random mutagenesis of the ABC transporter system. *Journal of Biotechnology.* 150 (4),453-459.

Lv, J., Huang, C., Zhang, X. and Tan, S. (2012). Extracellular secretion of anticoagulant peptide hirudin in *Lactococcus lactis* using SP310mut2 signal peptide. *Biotechnology Letters.* 34 (1),61-65.

Madsen, S. M., Arnau, J., Vrang, A., Givskov, M. and Israelsen, H. (1999). Molecular characterization of the pH-inducible and growth phase-dependent promoter P170 of *Lactococcus lactis*. *Molecular Microbiology.* 32 (1),75-87.

Makrides, S. C. (1996). Strategies for achieving high-level expression of genes in *Escherichia coli*. *Microbiological Reviews.* 60 (3),512-38.

Martinez-Alonso, M., Garcia-Fruitos, E., Ferrer-Miralles, N., Rinas, U. and Villaverde, A. (2010). Side effects of chaperone gene co-expression in recombinant protein production. *Microbial Cell Factories.* 9 (1),64.

Mathiesen, G., Sveen, A., Piard, J. C., Axelsson, L. and Eijsink, V. G. H. (2008). Heterologous protein secretion by *Lactobacillus plantarum* using homologous signal peptides. *Journal of Applied Microbiology.* 105 (1),215-226.

Mergulhão, F. J. M., Summers, D. K. and Monteiro, G. A. (2005). Recombinant protein secretion in *Escherichia coli*. *Biotechnology Advances.* 23 (3),177-202.

Mierau, I., Olieman, K., Mond, J. and Smid, E. (2005). Optimization of the *Lactococcus lactis* nisin-controlled gene expression system NICE for industrial applications. *Microbial Cell Factories.* 4 (1),16.

Miyoshi, A., Jamet, E., Commissaire, J., Renault, P., Langella, P. and Azevedo, V. 2004. *A xylose-inducible expression system for Lactococcus lactis*.

Miyoshi, A., Bermudez-Humaran, L. G., Ribeiro, L. A., Le Loir, Y., Oliveira, S. C., Langella, P. and Azevedo, V. (2006). Heterologous expression of Brucella

abortusGroEL heat-shock protein in *Lactococcus lactis*. *Microbial Cell. Factories*. (5) 14.

Morello, E., Bermúdez-Humarán, L. G., Llull, D., Solé, V., Miraglio, N., Langella, P. and Poquet, I. (2008). *Lactococcus lactis*, an Efficient Cell Factory for Recombinant Protein Production and Secretion *Journal of Molecular Microbiology and Biotechnology.* 14 48-58.

Morello, E., Poquet, I., Langella, P. and Flickinger, M. C.(2009). Secretion of Heterologous Proteins, Gram-Positive Bacteria, *Lactococcus lactis*. *Encyclopedia of Industrial Biotechnology.* John Wiley & Sons, Inc.

Nesmeyanova, M. A., Karamyshev, A. L., Karamysheva, Z. N., Kalinin, A. E., Ksenzenko, V. N. and Kajava, A. V. (1997). Positively charged lysine at the N-terminus of the signal peptide of the *Escherichia coli* alkaline phosphatase provides the secretion efficiency and is involved in the interaction with anionic phospholipids. *FEBS Letters.* 403 (2),203-207.

Ng, D. T., Brown, J. D. and Walter, P. (1996). Signal sequences specify the targeting route to the endoplasmic reticulum membrane. *The Journal of Cell Biology.* 134 (2),269-278.

Ng, D. T. W. and Sarkar, C. A. (2011). Nisin-inducible secretion of a biologically active single-chain insulin analog by *Lactococcus lactis* NZ9000. *Biotechnology and Bioengineering.* 108 (8),1987-1996.

Ng, D. T. W. and Sarkar, C. A. (2012). Engineering Signal Peptide for Enhanced Protein Secretion in *Lactococcus lactis*. *Applied and Environmental Microbiology.*

Noreen, N., Hooi, W., Baradaran, A., Rosfarizan, M., Sieo, C., Rosli, M., Yusoff, K. and Raha, A. (2011). *Lactococcus lactis* M4, a potential host for the expression of heterologous proteins. *Microbial Cell Factories.* 10 (1),28.

Nouaille, S., Morello, E., Cortez-Peres, N., Le Loir, Y., Commissaire, J., Gratadoux, J. J., Poumerol, E., Gruss, A. and Langella, P. (2006). Complementation of the *Lactococcus lactis* Secretion Machinery with Bacillus subtilis SecDF Improves Secretion of *Staphylococcal* Nuclease. *Applied and Environmental Microbiology.* 72 (3),2272-2279.

Nouaille, S., Bermudez-Humaran, L. G., Adel-Patient, K., Commissaire, J., Gruss, A., Wal, J. M., Azevedo, V., Langella, P. and Chatel, J. M. 2005

Improvement of bovine beta-lactoglobulin production and secretion by *Lactococcus lactis*. Braz. J. Med. Biol. Res. (38) 3: 353-359.

Oddone, G. M., Lan, C. Q., Rawsthorne, H., Mills, D. A. and Block, D. E. (2007). Optimization of fed-batch production of the model recombinant protein GFP in *Lactococcus lactis*. *Biotechnology and Bioengineering.* 96 (6),1127-1138.

Oguro, A., Kakeshita, H., Honda, K., Takamatsu, H., Nakamura, K. andYamane, K. (1995). srb: a Bacillus subtilis Gene Encoding a Homologue of the α-Subunit of the Mammalian Signal Recognition Particle Receptor. *DNA Research,* 2**,** 95-100.

Ong, R., Goh, K., Mahadi, N., Hassan, O., Rahman, R. and Illias, R. (2008). Cloning, extracellular expression and characterization of a predominant β-CGTase from Bacillus sp. G1 in *E. coli*. *Journal of Industrial Microbiology & Biotechnology.* 35 (12),1705-1714.

Pavan, S., Hols, P., Delcour, J., Geoffroy, M.-C., Grangette, C., Kleerebezem, M. and Mercenier, A. (2000). Adaptation of the Nisin-Controlled Expression System in *Lactobacillus plantarum*: a Tool To Study In Vivo Biological Effects. *Applied and Environmental Microbiology.* 66 (10),4427-4432.

Pearson, M. S., McManus, D. P., Smyth, D. J., Lewis, F. A. andLoukas, A. (2005). In vitro and in silico analysis of signal peptides from the human blood fluke, Schistosoma mansoni. *FEMS Immunology & Medical Microbiology,* 45**,** 201-211.

Poquet, I., Ehrlich, S. D. and Gruss, A. (1998). An Export-Specific Reporter Designed for Gram-Positive Bacteria: Application to *Lactococcus lactis*. *Journal of Bacteriology.* 180 (7),1904-1912.

Poquet, I., Saint, V., Seznec, E., Simoes, N., Bolotin, A. and Gruss, A. (2000). HtrA is the unique surface housekeeping protease in *Lactococcus lactis* and is required for natural protein processing. *Molecular Microbiology.* 35 (5),1042-1051.

Raha, A., Varma, N., Yusoff, K., Ross, E. and Foo, H. (2005). Cell surface display system for *Lactococcus lactis*: a novel development for oral vaccine. *Applied Microbiol Biotechnology.* 68 75 - 81.

Rahman, R. N. Z. R. A., Leow, T. C., Basri, M. and Salleh, A. B. (2005). Secretory expression of thermostable T1 lipase through bacteriocin release protein. *Protein Expression and Purification.* 40 (2),411-416.

Ravn, P., Arnau, J., Madsen, S. M., Vrang, A. and Israelsen, H. (2000). The development of TnNuc and its use for the isolation of novel secretion signals in *Lactococcus lactis*. *Gene.* 242 (1–2),347-356.

Ravn, P., Arnau, J., Madsen, S. M., Vrang, A. and Israelsen, H. (2003). Optimization of signal peptide SP310 for heterologous protein production in *Lactococcus lactis*. *Microbiology.* 149 (8),2193-2201.

Ribeiro, L., Azevedo, V., Le Loir, Y., Oliveira, S., Dieye, Y., Piard, J., Gruss, A. and Langella, P. (2002). Production and targeting of the *Brucella abortus* antigen L7/L12 in *Lactococcus lactis*: a first step towards food-grade live vaccines against brucellosis. *Applied Environment Microbiology.* 68 910 - 916.

Riesenberg, D. and Guthke, R. (1999). High-cell-density cultivation of microorganisms. *Applied Microbiology and Biotechnology.* 51 (4),422-430.

Sahdev, S., Khattar, S. and Saini, K. (2008). Production of active eukaryotic proteins through bacterial expression systems: a review of the existing biotechnology strategies. *Molecular and Cellular Biochemistry.* 307 (1),249-264.

Samaržija, D., Antunic, N. and and Havranek, J. K. (2001). Taxonomy, physiology and growth of *Lactococcus lactis*: a review. *Mljekarstvo* 51 (1),35-48.

Sambrook, J., Fritsch, E. and Maniatis, T. (1989). Molecular cloning: a laboratory manual.

Schotte, L., Steidler, L., Vandekerckhove, J. and Remaut, E. (2000). Secretion of biologically active murine interleukin-10 by *Lactococcus lactis*. *Enzyme and Microbial Technology*, 27**,** 761-765.

Sian, H. K., Said, M., Hassan, O., Kamaruddin, K., Ismail, A. F., Rahman, R. A., Mahmood, N. A. N. and Illias, R. M. (2005). Purification and characterization of cyclodextrin glucanotransferase from alkalophilic *Bacillus sp. G1*. *Process Biochemistry.* 40 (3–4),1101-1111.

Sirén, N., Salonen, K., Leisola, M. and Nyyssölä, A. (2008). A new and efficient phosphate starvation inducible expression system for *Lactococcus lactis* *Applied Microbiology and Biotechnology.* 79 (5),803-810.

Sirén, N., Salonen, K., Leisola, M. and Nyyssölä, A. (2009). A new salt inducible expression system for *Lactococcus lactis*. *Biochemical Engineering Journal.* 48 (1),132-135.

Sriraman, K. and Jayaraman, G. (2008). HtrA Is Essential for Efficient Secretion of Recombinant Proteins by *Lactococcus lactis*. *Applied Microbiology and Biotechnology*. 74 (23),7442-7446.

Steidler, L., Neirynck, S., Huyghebaert, N., Snoeck, V., Vermeire, A., Goddeeris, B., Cox, E., Remon, J. P. and Remaut, E. (2003). Biological containment of genetically modified *Lactococcus lactis* for intestinal delivery of human interleukin 10. Nature Biotechnology. 21(7),785-789.

Subramaniam, M.,Badaran, A.,Rosli, M.I.,Rosfarizan, M.,Yusoff, K.,and Rahim, R.A.(2012). Effect of Signal Peptides on the Secretion of Cyclodextrin Glucanotransferase in *Lactococcus lactis*NZ9000. *Journal of Molecular Microbiol Biotechnology*. 22, 361–372

Suciu, D. and Inouye, M. (1996). The 19-residue pro-peptide of staphylococcal nuclease has a profound secretion-enhancing ability in Escherichia coli. *Molecular Microbiology,* 21**,** 181-195.

Sunitha, K., Kim, Y.-O., Lee, J.-K. and Oh, T.-K. (2000). Statistical optimization of seed and induction conditions to enhance phytase production by recombinant *Escherichia coli*. *Biochemical Engineering Journal.* 5 (1),51-56.

Tjalsma, H., Antelmann, H., Jongbloed, J. D. H., Braun, P. G., Darmon, E., Dorenbos, R., Dubois, J.-Y. F., Westers, H., Zanen, G., Quax, W. J., Kuipers, O. P., Bron, S., Hecker, M. and van Dijl, J. M. (2004). Proteomics of Protein Secretion by *Bacillus subtilis:* Separating the "Secrets" of the Secretome. *Microbiology and Molecular Biology Reviews.* 68 (2),207-233.

Tjalsma, H., Bolhuis, A., Jongbloed, J. D. H., Bron, S. and van Dijl, J. M. (2000). Signal Peptide-Dependent Protein Transport in *Bacillus subtilis*: a Genome-Based Survey of the Secretome. *Microbiology and Molecular Biology Reviews.* 64 (3),515-547.

Tonkova, A. (1998). Bacterial cyclodextrin glucanotransferase. *Enzyme and Microbial Technology.* 22 (8),678-686.

van Asseldonk, M., Simons, A., Visser, H., de Vos, W. M. and Simons, G. (1993). Cloning, nucleotide sequence, and regulatory analysis of the *Lactococcus lactis* dnaJ gene. *Journal of Bacteriology.* 175 (6),1637-1644.

van Asseldonk, M., Rutten, G., Oteman, M., Siezen, R. J., de Vos, W. M. and Simons, G. (1990). Cloning of usp45, a gene encoding a secreted protein from *Lactococcus lactis* subsp. lactis MG1363. *Gene.* 95**,** 155-160.

van der Vossen, J. M., van der Lelie, D. and Venema, G. (1987). Isolation and characterization of *Streptococcus cremoris* Wg2-specific promoters. *A Applied Microbiology and Biotechnology.* 53 (10),2452-2457.

van Roosmalen, M. L., Geukens, N., Jongbloed, J. D. H., Tjalsma, H., Dubois, J.-Y. F., Bron, S., van Dijl, J. M. and Anné, J. (2004). Type I signal peptidases of Gram-positive bacteria. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research.* 1694 (1–3),279-297.

van Wely, K. H. M., Swaving, J., Freudl, R. and Driessen, A. J. M. (2001). Translocation of proteins across the cell envelope of Gram-positive bacteria. *FEMS Microbiology Reviews.* 25 (4),437-454.

Veenendaal, A. K. J., van der Does, C. and Driessen, A. J. M. (2004). The protein-conducting channel SecYEG. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research.* 1694 (1–3),81-95.

Vidgrén, G., Palva, I., Pakkanen, R., Lounatmaa, K. and Palva, A. (1992). S-layer protein gene of *Lactobacillus brevis*: cloning by polymerase chain reaction and determination of the nucleotide sequence. *Journal of Bacteriology.* 174 (22),7419-7427.

Viegas, S. C., Fernández de Palencia, P., Amblar, M., Arraiano, C. M. and López, P. (2004). Development of an inducible system to control and easily monitor gene expression in *Lactococcus lactis*. *Plasmid.* 51 (3),256-264.

von Heijne, G. and Abrahmsèn, L. (1989). Species-specific variation in signal peptide design Implications for protein secretion in foreign hosts. *FEBS Letters.* 244 (2),439-446.

Wahlström, E., Vitikainen, M., Kontinen, V. P. and Sarvas, M. (2003). The extracytoplasmic folding factor PrsA is required for protein secretion only in the presence of the cell wall in *Bacillus subtilis*. *Microbiology.* 149 (3),569-577.

Wang, Y.-h., Jing, C.-f., Yang, B., Mainda, G., Dong, M.-l. and Xu, A.-l. (2005). Production of a new sea anemone neurotoxin by recombinant *Escherichia coli*: Optimization of culture conditions using response surface methodology. *Process Biochemistry.* 40 (8),2721-2728.

Wegmann, U., O'Connell-Motherway, M., Zomer, A., Buist, G., Shearman, C., Canchaya, C., Ventura, M., Goesmann, A., Gasson, M. J., Kuipers, O. P., van Sinderen, D. and Kok, J. (2007). Complete Genome Sequence of the

Prototype Lactic Acid Bacterium *Lactococcus lactis* subsp. cremoris MG1363. *Journal of Bacteriology.* 189 (8),3256–3270.

Wieczorek, A. and Martin, V. (2010). Engineering the cell surface display of cohesins for assembly of cellulosome-inspired enzyme complexes on *Lactococcus lactis*. *Microbial Cell Factories.* 9 (1),69.

Zanen, G., Houben, E. N. G., Meima, R., Tjalsma, H., Jongbloed, J. D. H., Westers, H., Oudega, B., Luirink, J., van Dijl, J. M. and Quax, W. J. (2005). Signal peptide hydrophobicity is critical for early stages in protein export by *Bacillus subtilis*. *FEBS Journal*. 272 (18),4617-4630.

Zhou, X. X., Wang, Y. B., Pan, Y. J. and Li, W. F. (2008). Nisin-controlled extracellular production of apidaecin in *Lactococcus lactis*. *Applied Microbiol Biotechnology*. 78(6),947-953.